Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature (T_m) of a perfect hybrid. However, as the length of the probe is decreased, the T_m is lowered to the point where it is often impractical to carry out hybridization at $T_m - 25$°C. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the T_m. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the T_m are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the T_m are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the T_m) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated T_m of the hybrid. For oligonucleotides shorter than 18 nucleotides, the T_m of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the T_m of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the T_m of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - (600/N),$$

where N = chain length, predicts reasonably well the T_m for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

ESTIMATING THE EFFECTS OF MISMATCHES

Perhaps surprisingly, the classic formula (Bonner et al. 1973) to calculate the effect of mismatches on the stability of long DNA hybrids holds reasonably well for hybrids involving short oligonucleotides: For every 1% of mismatching of bases in a double-stranded DNA, there is a reduction of T_m by 1–1.5°C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and, even more critically, on the distribution of mismatched bases in the double-stranded DNA. Mismatches in the middle of the oligonucleotide are far more deleterious than mismatches at the ends. Therefore, the method of estimation given above should only be used as a rough guide until a systematic study of all types of mismatches in a variety of contexts leads to more precise methods of estimation. If appropriate target DNA has been cloned, the effect of mismatches on T_m can be determined empirically (see pages 11.55–11.57).

HYBRIDIZATION OF POOLS OF OLIGONUCLEOTIDES

It is easy to calculate accurately the T_m of a perfectly matched hybrid formed between a single oligonucleotide and its target sequence. However, when using pools of oligonucleotides whose members have greatly different contents of G + C, it is impossible to estimate a consensus T_m. Because it is not possible to know which member of the pool will match the target sequence perfectly, conditions must be used that allow the oligonucleotide with the lowest content of G + C to hybridize efficiently. Usually, conditions are chosen to be 2°C below the calculated T_m of the most A/T-rich member of the pool (Suggs et al. 1981b). However, the use of such "lowest common denominator" conditions can lead to a number of false positives, because mismatched hybrids formed by oligonucleotides of higher G + C content may be more stable than a perfectly matched hybrid formed by the correct oligonucleotide. In most cases, this problem is not serious, since the number of positive clones obtained by screening cDNA libraries with pools of oligonucleotides is usually quite manageable. It is therefore possible to easily distinguish false positives from true positives by another test (e.g., DNA sequencing or hybridization with a second pool of oligonucleotides corresponding to another segment of amino acid sequence).

In those cases when the number of positives is unacceptably high, it may be worthwhile to consider using hybridization solvents that contain the quaternary alkylammonium salts tetraethylammonium chloride (TEACl) or tetramethylammonium chloride (TMACl) instead of sodium chloride (Melchior and von Hippel 1973; Jacobs et al. 1985, 1988; Wood et al. 1985; Gitschier et al. 1986; Wozney 1989). In these solvents, the T_m of a hybrid is independent of its base composition and dependent primarily on its length. Thus, by choosing a temperature for hybridization appropriate for the lengths of the oligonucleotides in a pool, the effects of potential mismatches can be minimized.

It is important to obtain an accurate estimate of the T_m in TMACl or TEACl before using pools of oligonucleotides to screen cDNA or genomic DNA libraries. Jacobs et al. (1988) measured the T_i (the irreversible melting temperature of the hybrid formed between the probe and its target sequence) as a function of chain length for a number of oligonucleotides of differing G + C content in solvents containing either sodium or tetramethylammonium ions. Hybrids involving oligonucleotides 16 and 19 nucleotides in length melt over a smaller range of temperature in solvents containing TMACl than in solvents containing sodium salts (3°C for TMACl vs. 17°C for SSC when hybridizing 16-mers; 5°C for TMACl vs. 20°C for SSC when hybridizing 19-mers). For 14-mers, the effect is much less dramatic (7°C for TMACl vs. 9°C for SSC). Similar, but less extensive, data are available for solvents containing TEACl (Jacobs et al. 1988).

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48–50°C; for 19-mers, it is 55–57°C; and for 20-mers, it is 58–66°C. Three points are worth emphasizing. First, the T_is of hybrids are uniformly 15–20°C higher in solvents containing TMACl than in solvents containing TEACl. The higher T_i in solvents containing TMACl allows hybridization to be performed at temperatures that

suppress nonspecific adsorption of the probe to solid supports (such as nylon membranes), resulting in lower nonspecific backgrounds. Second, hybridization solvents containing TMACl do not have significant advantages over those containing sodium ions until the length of the oligonucleotide exceeds 16 nucleotides. Finally, the data have been extensively examined for 16-mers, 19-mers, and, in previous studies, for much longer DNA molecules (Melchior and von Hippel 1973). It is currently an untested assumption that the same beneficial effect will be seen for DNA molecules of all intermediate lengths.

Preparation and Use of Solvents Containing Quaternary Alkylammonium Salts

1. Prepare a 6 M solution of tetramethylammonium chloride (TMACl) or a 3 M solution of tetraethylammonium chloride (TEACl) in H_2O. (TMACl and TEACl are available from Aldrich.)

2. Add activated charcoal to a final concentration of approximately 10% and stir for 20–30 minutes.

3. Allow the charcoal to settle, and then filter the solution of TMACl or TEACl through a Whatman No. 1 paper.

4. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). Store the filtered solution in dark bottles at room temperature.

5. Measure the refractive index of the solution, and calculate the precise concentration of the solution from the equation:

$$C = (n - 1.331)/0.018$$

where C = molar concentration of quaternary alkylammonium salt and n = refractive index.

6. Prehybridize nitrocellulose filters or nylon membranes for 2–6 hours in oligonucleotide prehybridization solution.

Oligonucleotide prehybridization solution
6× SSC (or 6 × SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

7. Prepare the quaternary alkylammonium solution to be used for hybridization:

Quaternary alkylammonium hybridization solution
3.0 M TMACl or 2.4 M TEACl
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 7.6)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
0.1% nonfat dried milk

Notes

i. Nitrocellulose filters are not stable when hybridization is carried out for extended periods of time in solvents containing TMACl or TEACl. Nylon membranes are much better suited for this purpose.

ii. Posthybridization washing is usually carried out initially with solutions containing sodium salts (e.g., 6 × SSC) rather than quaternary alkylammonium salts. If additional stringent washes are required, rinse the filters first with quaternary alkylammonium hybridization solution (without DNA or nonfat dried milk) at room temperature and then briefly (5–10 minutes) with the same solution at $T_i - 5°C$.

HYBRIDIZATION OF GUESSMERS

Perhaps the most critical step in the use of guessmers is the choice of conditions for hybridization. The temperature should be high enough to suppress hybridization of the probe to incorrect sequences but must not be so high as to prevent hybridization to the correct sequence, even though it may be mismatched. Before using an oligonucleotide to screen a library, it is therefore advisable to perform a series of trial experiments in which a series of northern or genomic Southern hybridizations are carried out under different degrees of stringency (Anderson and Kingston 1983; Wood et al. 1984). A set of theoretical curves relating the temperature of the washing solution to the length and homology of the probe is given in Lathe (1985). Using these curves as a guide, determine the optimal conditions for detection of sequences complementary to the probe by hybridizing the oligonucleotide to a series of nitrocellulose filters or nylon membranes at different temperatures. The filters are washed extensively in 6× SSC at room temperature and then briefly (5–10 minutes in 6× SSC) at the temperature used for hybridization. This method, in which both hybridization and washing are carried out under the same conditions of temperature and ionic strength, appears to be more discriminating than the more commonly used procedure of hybridizing under conditions of lower stringency and washing under conditions of higher stringency.

If trial experiments are not possible, attempt to estimate the melting temperature (T_m) as follows:

1. Calculate the minimum G + C content of the oligonucleotide assuming that A or T is present at all positions of ambiguity.
2. Using the following formula, calculate the T_m of a double-stranded DNA with the calculated G + C content:

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G + C) - (600/N)$$

where N = chain length.

3. Calculate the maximum amount of possible mismatch assuming that all choices of degenerate codons are incorrect. Subtract 1°C from the calculated T_m for each 1% of mismatch. The resulting number should be the T_m of a maximally mismatched hybrid formed between the probe and its target DNA sequence.

In the absence of information from trial experiments, hybridization and washing should be carried out at 5–10°C below the estimated T_m. Almost certainly, the actual T_m will be higher than that predicted by this worst-case calculation. If the bases used at positions of ambiguity were chosen at random, one out of four should be correct, and approximately half of these would be expected to be G or C. The observed T_m should therefore be significantly higher than that estimated. However, to minimize the risk of missing the clone of interest, it is best to hybridize and wash at several degrees below the T_m estimated as described above. If, under these conditions, the probe hybridizes indiscriminately, repeat the hybridization at a higher temperature or wash under conditions of higher stringency.

Before proceeding to screen an entire cDNA or genomic DNA library, it is advisable to carry out a series of pilot experiments in which the probe is hybridized under different conditions to small aliquots (perhaps 5000–10,000 clones) of the library that is to be screened. The results of these experiments should allow you to choose conditions for large-scale screening that are just stringent enough to eliminate nondiscriminate hybridization of the probe to the vast majority of clones in the library.

Hybridization of guessmers in solvents that contain quaternary alkylammonium salts has not been investigated.

HYBRIDIZATION OF OLIGONUCLEOTIDES THAT CONTAIN A NEUTRAL BASE AT POSITIONS OF DEGENERACY

Although the conditions for hybridization of probes that contain the neutral base inosine have not been extensively explored, it is possible to make a conservative estimate of the melting temperature (T_m) as follows:

1. Subtract the number of inosine residues from the total number of nucleotides in the probe to give a value S.
2. Calculate the G + C content of S.
3. Estimate the T_m of a perfect hybrid involving S using the equation on page 11.52.
4. Use conditions for hybridization that are 15–20°C below the estimated T_m.

The T_m of hybrids involving oligonucleotides that contain neutral bases can also be estimated empirically as described on pages 11.55–11.57. Hybridization of such oligonucleotides in solvents containing quaternary alkylammonium salts has not been investigated.

EMPIRICAL DETERMINATION OF MELTING TEMPERATURE

The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be determined by the procedure described below. The protocol actually measures the temperature at which dissociation of the double-stranded DNA becomes irreversible (T_i) in nonequilibrium conditions that do not favor rehybridization of the released probe to the target. The optimal temperature for hybridization is then determined on the basis of this value. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. In most cases, a target sequence is not available from "natural" sources and must be synthesized chemically. The best synthetic target sequences consist of two oligonucleotides that are partially complementary. After annealing, these oligonucleotides form a double-stranded region that contains the target sequence. The sequences of the protruding ends are designed to allow the target DNA to be cloned easily in bacteriophage M13 vectors. Single-stranded DNA of the appropriate orientation prepared from the resulting clones (see Chapter 4) can be used in hybridization experiments as described below. It can also be used as a template for dideoxy-mediated chain-termination sequencing (see Chapter 13) if it is necessary to check that the sequence of the target DNA is correct.

1. Label 1–10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (see pages 11.31–11.32), and remove excess unincorporated [γ-^{32}P]ATP by one of the methods described on pages 11.33–11.39.

2. Using a paper punch, prepare four small circles (diameter 3–4 mm) of a solid support (nitrocellulose filter or nylon membrane) used for hybridization. Arrange the small circles on a piece of Parafilm. Mark two of the filters with a soft-lead pencil.

3. Apply approximately 100 ng of target single-stranded DNA in a volume of 1–3 μl of 2× SSC to each of the marked filters. Apply an equal amount of vector DNA to the unmarked filters. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of filters from the Parafilm, and place them between sheets of Whatman 3MM paper. Fix the DNAs to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   If the target DNA has been cloned into a plasmid, linearize the vector by digestion with a restriction enzyme that does not cleave within the target sequences. Purify the resulting double-stranded DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 2× SSC at a concentration of 500 ng/μl. Apply the solution of DNA to the filters prepared as described above, and then, using blunt-ended forceps, transfer the filters to a sheet of 3MM paper saturated with denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5–10 minutes. Move the filters to a fresh sheet of 3MM paper saturated with neutralizing solution (0.5 M Tris · Cl [pH 7.4], 1.5 M NaCl) for 10 minutes. Transfer the filters to a dry sheet of 3MM paper, and leave them at room temperature until all of the fluid has evaporated. Bake the filters as described above.

   Overbaking can cause the filters to become brittle. In addition, filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

4. Using blunt-ended forceps, transfer all of the filters to a polyethylene tube that contains 2 ml of oligonucleotide prehybridization solution. Seal the tube and incubate, with occasional shaking, at a temperature estimated to be $T_m - 25°C$ for the solvent being used (see Note i). After 2 hours, add radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approximately 1 pmole/ml. Continue incubation at $T_m - 25°C$ for a further 2–4 hours, with occasional shaking.

Oligonucleotide prehybridization solution
6× SSC (or 6× SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

5. Remove the filters from the hybridization solution, and immediately immerse them in 2× SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the filters remains constant (as measured with a hand-held minimonitor).

6. Adjust the temperature of a circulating water bath to $T_m - 25°C$. Dispense 5 ml of 2× SSC into each of 20 glass test tubes (17 mm × 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2× SSC is $T_m - 25°C$. The 2× SSC in each of these tubes will be used separately for each temperature increase (see steps 7–10).

7. Transfer the filters individually to four empty glass tubes, separating the filters containing the vector and target DNAs, and add 1 ml of 2× SSC (from one of the tubes prepared in step 6 and prewarmed to $T_m - 25°C$). Place the tubes in the water bath for 5 minutes.

8. Remove the tubes from the bath, transfer the liquid to scintillation vials, and wash the tubes and filters with 1 ml of 2× SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.

9. Increase the temperature of the water bath by 3°C, and wait for the temperature of the 2× SSC in the tubes prepared in step 6 to equilibrate.

10. Add 1 ml of 2× SSC at the higher temperature to each of the four tubes containing the filters. Place the tubes in the water bath for 5 minutes.

11. Repeat steps 8, 9, and 10 at successively higher temperatures until a temperature of $T_m + 30°C$ is achieved.

12. Place the filters in separate glass tubes (17 mm × 100 mm) containing 1 ml of 2 × SSC, and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the filters and tubes used for boiling with 1 ml of 2 × SSC, and add the washing solutions to the appropriate scintillation vials.

13. Use a scintillation counter to measure the radioactivity (by Cerenkov counting, see Appendix E) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature (i.e., the sum of radioactivity eluted at all temperatures between $T_m - 25°C$ and the temperature at which a given sample was taken divided by the total radioactivity eluted from the filters at all temperatures up to and including 100°C).

    If the experiment has worked well, very little radioactivity should be associated with the filters containing vector DNA alone. Furthermore, this radioactivity should be completely released from the filters at temperatures much lower than the estimated T_m. On the other hand, considerable radioactivity should be associated with the filters containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the filters until a critical temperature is reached, and then approximately 90% of the radioactivity should be released during the succeeding 6–9°C rise in temperature. The temperature at which 50% of the radioactivity has eluted from the filters containing the target sequences is defined as the T_i of the hybrid between the probe and its target sequence.

Notes

i. Although the above protocol calls for the use of sodium salts in the solvent used for hybridization, other solutes such as tetramethylammonium chloride or tetraethylammonium chloride can be substituted if desired to determine the T_i in these solvents.

ii. This method can easily be adapted to study the behavior of hybrids formed between probes and target sequences that do not match each other perfectly (Jacobs et al. 1988).

iii. Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and the sequence of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search commonly used vectors for sequences that match the sequence of the probe closely enough to cause problems during hybridization.

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